Speeding the Gene Hunt: High-Speed DNA Sequencing

FAST. Faster. Fastest. In the commercial arena, getting big jobs done fast requires automation, For the Human Genome Project at Lawrence Livermore National Laboratory, the key to uncovering thousands of yet-to-be-identified human genes is to automate and speed up the specialized biotechnical equipment that prepares and sequences DNA samples.

The point of all this urgency is the gold mine of information contained within the structure of the genes themselves. Genes and the proteins they produce hold the key to unlocking the mysteries of genetic diseases. Once the genetic code for a disease is understood, researchers can begin developing gene and drug therapies for that particular disease. The ultimate goal of the worldwide Human Genome Project is to find all the genes in the DNA sequence, develop tools for using this information in the study of human biology and medicine, and improve human health.

Sequencing involves determining the exact order of the four individual chemical building blocks, or bases, that form DNA. The total DNA in a single human cell has approximately 3 billion pairs of the chemical building blocks adenine, thymine, guanine, and cystosine. (For more information on the Laboratory's other work in DNA sequencing, see *S&TR*, November 1996, pp. 24–26.)

For a multidisciplinary team of engineers, chemists, computer scientists, and biologists at Livermore, Joe Balch is project leader for developing a next-generation instrument for sequencing DNA. When this high-throughput DNA sequencer is built and its operating conditions are optimized, it will ultimately read nearly 600,000 bases per eight-hour shift, about 12 times faster than current instruments, which manage at most 48,000 bases per shift.

"There is a worldwide push in the field to 'pick up the speed' with which DNA is sequenced," said Balch. "The current strategy is to do it with existing technology and just turn the crank a lot of times with more people. It's very people-intensive. The next-generation sequencing machine we are developing will allow us to leave the old technology behind and take the next step in automation." Livermore expertise in microfabrication, bioinformatics, and biochemistry makes this move possible.

Faster sequencing will also provide other Livermore programs with faster access to information in nonproliferation projects to detect biological signatures of collected samples and in bioremediation projects to optimize micro-organism action.

Figure 1. Computer-generated image of fluorescent bands after the fragments are detected by the laser.

Sequencing: How It Works

When biological researchers want to sequence a section of DNA, they clone fragments of that section and then run four nearly identical reactions on those fragments. In these reactions, the four bases are chemically labeled with four different fluorescent dyes.

The sequencing machines currently used at the Laboratory are based on a gel-electrophoresis system, which works like this. The DNA samples are loaded by hand into a 200- to 400-micrometer-thick polyacrylamide gel, rather like thin Jello, which is sandwiched between two large glass plates, 48 centimeters long by 25 centimeters wide. The plates can hold 36 samples at a time. An electric current is then applied to the gel, and, because the DNA itself has a negative charge, the fragments migrate in 36 columns or "lanes" from the top to the bottom of the plate. The DNA fragments move at different rates depending on their size: smaller ones move faster than larger ones. As the fragments migrate past a certain point in the gel, a laser beam scans back and forth across the plate, exciting the dyes on the DNA bases. As the fragments pass the laser, the bases are separated from smallest to largest. The fluorescent signals generated by the laser are detected by photomultiplier tubes (or other detectors), and a computer captures, stores, and processes them (Figure 1).

When cleaning, loading, and running times are all taken into account, it takes between five to seven hours to complete a run. Each sample contains about 500 bases, which means each run of 36 samples yields no more than 18,000 bases. According to

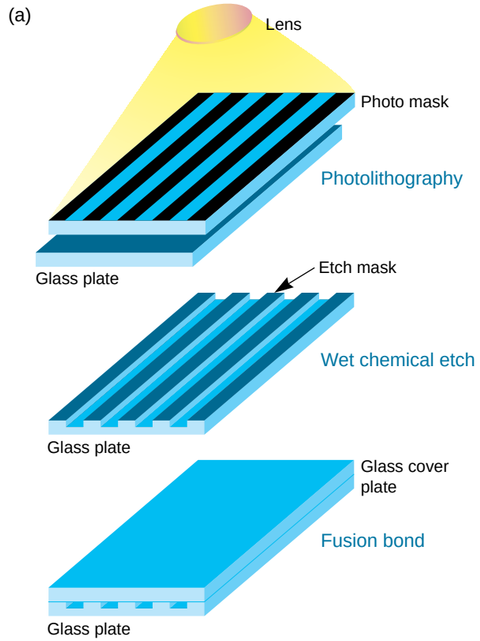

Figure 2. During fabrication of the new 96-lane sequencing machine, the pattern of channels is first defined by (a) photolithographic processing on a photoresist plate. (b) Then, that plate is used to chemically etch the pattern on the glass. (c) Finally, the top piece of glass is bonded to the etched glass plate.

Balch, conventional technology is expanding the number of lanes to 64, which will increase the yield to about 32,000. But to increase those numbers significantly, say, by an order of magnitude, requires applying some new technologies.

There are a number of ways to increase this yield, explained Balch, who is also the former head of the Laboratory's Microtechnology Center. (For an overview of the Center, see pp. 11–17 of this issue). "You can increase the number of lanes on a single run. You can increase the speed at which you do a run—in other words, apply more electric field to the fragments. You can also look for ways to cut down on the loading and cleanup times, which often take a couple of hours. But to do all of these things, you need to move outside the current technologies and look for different ways to get the job done." That is what they are doing for their new sequencing machine (Figure 2).

Increase the Lanes

In the current system, although the samples travel in "lanes," no physical barriers divide one lane from the next. "And even though you have an electric field pulling the fragments to the bottom, they still wander a bit," said Balch. "Right now, we correct the wandering with software, with what is called 'lane tracking.' But if we start packing more lanes in, there's a problem with the columns blurring into each other."

So Balch and his team are taking a different tack: fabricating small, exact lanes, or microchannels, in large glass plates through which the gel medium flows. This effort got its start in 1993 with seed money from the Lawrence Livermore's Laboratory Directed Research and Development Program. In 1994, the Laboratory entered a year-and-a-half agreement with Perkin Elmer's Applied Biosystems Division to further develop this technology and some of the others

needed for the new system. This effort is now being supported by grants from the National Institutes of Health and the DOE Human Genome Project.

Last year, Steve Swierkowski and Courtney Davidson of the Microtechnology Center successfully demonstrated the fabrication of a 96-lane array on a piece of glass 7.5 centimeters wide and 55 centimeters long—in other words, twice the lanes that current technology can offer in less than a third of the space. Ultimately, the team will be producing plates with 384 channels.

"These high-density microchannel glass plates are the really novel piece of our instrument," noted Balch. "The fabrication process involves three steps, each of which we need to continue to build and improve upon." (See Figure 2a.)

The first is the photolithography step, where the pattern of the channels is defined on a photomask plate. The second involves using that plate to chemically etch that very small pattern on the glass to very exact specifications. The final step is bonding the top piece of glass to the etched glass plate. Figure 2c shows the current prototype instrument developed by Davidson, Larry Brewer, Joe Kimbrough and Ron Pastrone.

Increase the Speed

Another way to speed up the process is to increase the electric field. The velocity of the DNA increases proportionally. In the current system, however, just increasing the field leads to other problems.

A higher electric field increases the power dissipation, which increases the temperature in the sieving media, explained Balch. And when the gel heats up—and the DNA samples in it—thermal diffusion causes the fluorescent bands to spread out. The bands run into each other and can no longer be identified as individual and distinct bands. This problem can be significantly reduced by using a very thin gel (about 50 micrometers thick) or other sieving media in place of the polyacrylamide gel now being used. The thinner gel means that the temperature gradient across the width of the gel is smaller, and the thermal diffusion of the DNA fragment bands is less.

With this thin sieving media, the instrument can run with an electrical field three to four times higher than that used on the conventional instrument. Thus, the speed of the run increases by the same factor.

Decrease the Clean-Up Time

Another improvement involves using small syringe pumps to inject thin sieving media into the microchannels of the new instrument when a run begins and then automatically pumping it out when a run is complete. This procedure will significantly speed up the overall time it takes to complete a run.

"With the polyacrylamide gel now in use, you have to go through a lengthy preparation at the start of a run to make a fresh gel. Then at the end, you have to take the plates apart, remove the old gel, and clean the plates for the next run. With this new media, we just pump it in and out through channels and capillaries without removing the microchannel plate from the instrument," said Balch. "We figure that when the system is up and running, one run will take two to three hours from start to finish, compared with four to five hours using the polyacrylamide gel."

Putting It All Together

Because the new system has different performance specifications than the old, simply loading the new glass plates with the new medium into the old machine is not the only change.

For instance, because the DNA fragments are moving at a higher velocity when they come to the laser, the laser has to scan across the plates faster. In addition, given the 96 lanes now and the 384 to come in the future, Balch and his team are exploring several concepts for automatic sample loading.

Other systems being developed include the laser-induced fluorescent detection system, the fluidic and pumping system for the polymer medium, a temperature control system, and analysis software. "These improvements plus the microchannel plates themselves add up to seven major parts of the high-throughput DNA sequencer that we must eventually meld together," said Balch. "The final production system is still down the road. When it's ready, we plan to make it available to others within the Department of Energy and the human genome community."

— Ann Parker

Key Words: DNA sequencing, Human Genome Project, microchannel, polyacrylamide gel.

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The Human Genome Center's Internet home page is at http://www.llnl.gov/bbrp/genome/genome.html.
The Department of Energy's "Primer on Molecular Genetics" is located at http://www.gdb.org/Dan/DOE/intro.html.

Microbial Treatment of High Explosives

Figure 1. The pilot test system for biological treatment of high-explosive waste at DOE's Pantex Plant.

WHILE we may not want bugs crawling around our homes, some of them make remarkable little workers. Special micro-organisms that possess certain enzymes effectively degrade the pollutants in sewage at treatment plants. They also clean up soil contaminated by petroleum products. At closed gasoline stations, the large mounds of earth contaminated by leaking underground storage tanks are being composted by a variety of microbes that occur naturally in soils.

Microbial treatment of contaminated materials has become increasingly common. In 1989, Lawrence Livermore National Laboratory chemist John Knezovich and environmental scientist Jeffrey Daniels began a study with Professor Michael Stenstrom of the University of California, Los Angeles, an expert in wastewater treatment, to determine the feasibility of using similar, naturally occurring micro-organisms to treat materials contaminated with high explosives. The goal: to break down the hazardous compounds into nonhazardous units. The success of this inexpensive method could have wide ramifications. The Department of Energy's Pantex Plant in Amarillo, Texas, disassembles nuclear weapons as it reduces the size of the stockpile and generates high-explosive waste in the process. The Department of Defense must dispose of an even larger quantity of high explosives. Various international treaties require the demilitarization of large numbers of conventional weapons, all of which contain high explosives.

Both DOE and DoD use water and/or steam in the process of removing high explosives, resulting in large quantities of contaminated water. This water typically is run through activated carbon filters, which remove the high explosives and leave clean water, but the process contaminates the carbon filters. In fact, activated carbon laden with high explosives is considered a hazardous waste by the U.S. Environmental Protection Agency. To dispose of the carbon, the Pantex Plant has been burning it, but this disposal process is becoming unacceptable for environmental, health, and safety reasons. Some DoD facilities have been storing it, but this is only a short-term solution. The current best method for decontaminating the carbon is to heat it in a relatively expensive process called thermal regeneration, which requires shipment of the contaminated carbon to an offsite treatment facility.

Livermore's 1989 study, funded by the Laboratory Directed Research and Development Program, first demonstrated the feasibility of biologically treating the small quantities of high-explosive waste that are in wastewater produced at the Laboratory's high-explosives testing range at Site 300. The results of experiments were encouraging and led to the Livermore team developing, installing, and testing a pilot plant at Pantex in 1993 for the direct treatment of wastewater and the regeneration of contaminated carbon (Figure 1). Measurements at that plant have proved that naturally occurring micro-organisms can directly degrade RDX and HMX, the most commonly used high explosives within the DOE.

By late 1997, with funding from DOE and DoD, the project team will install another pilot plant at the Hawthorne Army Depot's Western Area Demilitarization Facility in Hawthorne, Nevada. That facility presently uses a steam-out process for removing high explosives from conventional ordnance, resulting in an annual production of up to 25 million gallons (95,000 kiloliters) of water contaminated with high explosives. Subsequent treatment of the wastewater by activated carbon filters generates approximately 120,000 pounds (130 metric tons) of contaminated carbon.

Bugs Need Help

Microbes that clean up sewage and gasoline-contaminated soil often directly feed on those materials. During the feasibility study, the project team learned that bugs do not "eat" the high explosives. Instead, a systematic process of supplying various nutrients showed that when the micro-organisms are fed ethanol and other simple compounds containing carbon in the presence of high explosives, they produce enzymes that degrade RDX and HMX.

Although the pilot plant at Pantex demonstrated that microbial treatment of water contaminated with high explosives was feasible, this process would not be efficient for the large quantities of wastewater that are generated at demilitarization facilities. Accordingly, the team developed a method that couples a chemical process that removes and degrades the high explosives on the carbon with subsequent biological treatment of the wastewater to render the by-products nonhazardous.

As illustrated in Figure 2, the procedure now in development involves first flushing the carbon-filled column with heated (80°C) alkaline water (pH ≥ 12). This process, known as base hydrolysis, regenerates the carbon by removing the trapped high explosives and transforms the explosive materials into nonexplosive but easily degraded hazardous carbon compounds. As the wastewater flows from the carbon column to the "bioreactor" column, ethanol and other nutrients are added to the mixture. The microbial action in the bioreactor rapidly completes the breakdown process to nonhazardous products.

All high explosives contain nitrogen, in the form of nitro-groups, nitrates, or nitramines. The team analyzed the compounds in the effluent from the bioreactor column and found that essentially complete denitrification of those compounds occurs. Ideally, denitrification would reduce the compounds to nitrogen gas only. In this case, over 98% of the gas released by the micro-organisms is nitrogen, and the remainder consists primarily of oxygen, carbon dioxide, and hydrogen, none of which is a hazardous material. In addition, the majority of carbon-containing molecules have been converted to carbon dioxide, with the remainder converted to low-molecular-weight fragments that are below detection limits. Studies conducted with high-explosive compounds tagged with carbon-14 have verified that microbial action has converted all of the materials to their smallest possible units, which is the goal of any microbial treatment.

The pilot plant at the Pantex facility included pilot-scale biological treatment and will add pilot-scale chemical regeneration of carbon by the end of the year. Results from laboratory experiments for chemical regeneration of carbon and operation of the Pantex pilot plant indicate that the coupled chemical and biological method of regenerating activated carbon laden with high explosives is feasible, effective, safe, and efficient. Preliminary calculations also show that this process should be significantly less expensive than thermal regeneration and can be performed on site. Thermal treatment of 120,000 pounds of carbon per year (the amount produced annually at the Hawthorne facility) is estimated to cost about $0.79 per pound, while the coupled chemical/biological method should cost from $0.21 to $0.30 per pound.

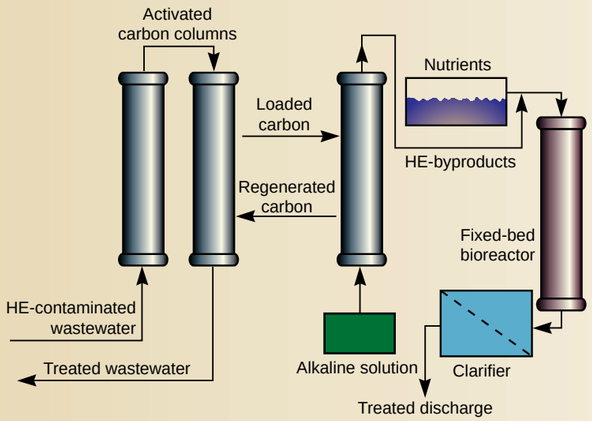

Figure 2. Schematic of the coupled system of carbon regeneration and biological and carbon treatment of high-explosive wastes.

The Next Step

The pilot plant at Pantex is relatively small because it was used to define the feasibility of the process and to determine the operating parameters for a larger treatment system. The bioreactor containing the micro-organisms is a column 1.7 meters (5.5 feet) high with an interior diameter of 0.18 meters (7 inches). The explosives-laden solution flows through the bioreactor just once at a rate of 60 milliliters per minute, staying in the bioreactor for about 8 hours.

A similarly sized pilot plant is planned for installation and operation at Hawthorne later this year. It will have a significantly greater capacity, however, because it will incorporate the carbon treatment via base hydrolysis, which is more efficient than biological treatment alone.

Knezovich, Daniels, Stenstrom, and their colleagues are expanding the process so that it could be applied to other high explosives. For example, ongoing research is addressing the treatment of TNT, in which the U.S. Army is particularly interested. Because TNT is more difficult for micro-organisms to degrade than RDX and HMX, the team is working to improve the base hydrolysis process to convert TNT to products that are more amenable to biological degradation. The results of this work will be used to optimize the treatment process at Hawthorne over the next two years. The team will also be looking at the feasibility of expanding these approaches for treatment of other high-explosive wastes.

— Katie Walter

Key Words: decontamination, demilitarization, hazardous waste, high explosives, microbial treatment.

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Patents and Awards

Each month in this space we report on the patents issued to and/or the awards received by Laboratory employees. Our goal is to showcase the distinguished scientific and technical achievements of our employees as well as to indicate the scale and scope of the work done at the Laboratory.

Patents

Patent issued to	Patent title, number, and date of issue	Summary of disclosure
Steven T. Mayer James L. Kaschmitter Richard W. Pekala	**Carbon Aerogel Electrodes for Direct Energy Conversion** U.S. Patent 5,601,938 February 11, 1997	A device, such as a fuel cell, that uses carbon–aerogel electrodes loaded with a noble catalyst, such as platinum or rhodium, and soaked with phosphoric acid. A separator is located between the electrodes, which are placed in a cylinder having plate current collectors positioned adjacent to the electrodes and connected to a power supply; a pair of gas manifolds, containing hydrogen and oxygen, is positioned adjacent the current collectors. Because of the high surface area and excellent electrical conductivity of carbon aerogels, problems are overcome regarding high-polarization resistance of carbon composite electrodes conventionally used in fuel cells.
Chi Y. Fu	**Laser Programmable Integrated Circuit for Forming Synapses in Neural Networks** U.S. Patent 5,602,965 February 11, 1997	A customizable network wherein all the resistors in the synaptic array are identical, thus simplifying processing. Doped, amorphous silicon is used as the resistor material to create extremely high resistances occupying very small spaces. Connected in series with each resistor in the array is at least one severable conductor whose uppermost layer has a lower reflectivity of laser energy than typical metal conductors at a particular laser wavelength. The neural-network-integrated chip may include a plurality of input isolation buffers for driving the input lines in the synaptic array and a plurality of neuron circuits receiving inputs from several of the synaptic-array output lines.
Thomas E. McEwan	**Electronic Multi-Purpose Material Level Sensor** U.S. Patent 5,609,059 March 11, 1977	A sensor based on time-domain reflectometry of very short pulses. Pulses are propagated along a transmission line or dipstick that is partially immersed in a liquid, powder, or other substance. A launcher plate can be used to help launch the pulses and to produce a fiducial pulse. The time difference of the reflections at the start of the transmission line and the air–material interface determine levels to about 1% accuracy. The low-cost sensor is essentially independent of circuit-element and temperature variations.
William J. Benett Peter A. Krulevitch Abraham P. Lee Milton A. Northrup James A. Folta	**Miniature Plastic Gripper and Fabrication Method** U.S. Patent 5,609,608 March 11, 1997	A gripper, constructed of either heat-shrinkable or heat-expandable plastic tubing, formed around a mandrel, then cut to form gripper prongs or jaws, and the mandrel removed. The gripper is connected at one end with a catheter having an actuating balloon at its tip, whereby the gripper is opened or closed by inflation or deflation of the balloon. The inexpensive gripper can be used for gripping, sorting, and placing of micrometer-size particles for analysis; endovascular release of embolic material for the treatment of neuro-aneurysms; and placement of small plugs into ovarian tubes for contraception.
Daniel W. Shimer Arnold C. Lange	**E-Beam High Voltage Switching Power Supply** U.S. Patent 5,610,452 March 11, 1997	A circuit coupled to a voltage source and a current source for limiting voltage spikes across two input circuit lines of a voltage-spike-producing circuit. The circuit consists of a capacitor having a first end connected to one input circuit line and first leads of the voltage and current sources; a resistor having a first end connected to the other end of the capacitor and a second end connected to second leads of the voltage and current sources; and a diode having an anode connected to the second end of the capacitor and a cathode connected to the other input circuit line and a third lead of the current source. Excess current flows from one input circuit line through the capacitor and diode to the other input circuit line, and voltage across the two circuit lines is clamped to the voltage source.